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Anne Holleran
AU: 1642
Tel: 308-8892
RM: 8e03

mailbox: 8e12

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Murine Immune Response to Cells Transfected with Human MUC1: Immunization with Cellular and Synthetic Antigens[1]

Vasso Apostolopoulos, Pei-Xiang Xing, and Ian F. C. McKenzie[2]

The Austin Research Institute, Studley Rd., Heidelberg 3084, Victoria, Australia

ABSTRACT

Humans with breast cancer have T-cells in their lymph nodes which recognize a peptide sequence within the variable number of tandem repeats of the mammary mucin, MUC1, which is overexpressed in breast cancer. To find means of making this recognition event into a potent immune response to breast cancer, we used a murine tumor model and have examined the parameters of the immune response to human mucin (MUC1) expressed in murine BALB/c 3T3 cells. We then sought to boost this response with MUC1-containing synthetic peptides, fusion proteins, and natural mucin (HMFG). MUC1$^+$3T3 cells were found to be rejected by BALB/c mice by day 15 due to a cellular [CD3$^+$, Ly2$^+$ (CD8$^+$)] response. The cellular rejection response was accompanied by the generation of CD8$^+$ cytotoxic T-cells, CD4$^+$ delayed-type hypersensitivity, and little anti-MUC1 antibody. This immune response is presumably of the TH_1 type (which occurs in CD8 as well as CD4 cells) of CD8$^+$ cytotoxic cells. By contrast, mice immunized with the MUC1 synthetic peptide, a fusion protein, or HMFG have good antibody responses, a delayed-type hypersensitivity reaction, but no cytotoxic T-cells and less tumor protection, possibly a TH_2 type response. We conclude that CD8$^+$ cytotoxic anti-mucin cells can produce significant antitumor responses *in vivo* to a human "tumor" antigen expressed in murine cells; immunization with soluble synthetic or native materials leads to the "humoral" (TH_2) type of immunity, and efforts need to be made to convert this to a TH_1-type response.

INTRODUCTION

Mucins are expressed in greater abundance in adenocarcinomas than in normal tissues, especially in the breast; therefore, monoclonal antibodies reacting with such mucins can give an apparent tumor specificity on tissue sections (1, 2). In addition, mammary cancer mucin has a ubiquitous rather than focal cellular distribution and may also have altered glycosylation, revealing peptide epitopes not easily identified in normal mucins (3, 4). These observations make mucins a potential target for the immunotherapy of adenocarcinomas because it is possible that such "hidden" self epitopes could be immunogenic in humans. Mucins are clearly immunogenic in mice; most monoclonal antibodies to carcinoma of the breast identify mucins, especially MUC1[3] (5). The cloning of the cDNA for MUC1 and the definition of the structure of the protein core indicates that most of the immunogenicity (with regard to antibody production) resides in a repeated (VNTR) 20-amino acid peptide (PDTRPAPGSTAPPAHGVTSA) domain in the extracellular portion of the molecule, and many anti-mucin antibodies made against tumor cells react with the amino acids APDTR within the repeat (6–8). We have extended these observations by making anti-peptide and anti-fusion protein antibodies to this APDTR sequence; such antibodies are not substantially different from the antitumor antibodies (9, 10). However, making mouse anti-peptide antibodies is not novel, and the observations cited above would have little relevance for tumor immunogenicity were it not for the findings of Barnd *et al.* (11) and others (12, 13) who demonstrated that in the lymph nodes of patients with breast cancer, cancer of the pancreas, ovary and other tumors are found cytotoxic lymphocytes which, after *in vitro* stimulation, react with human mucin. Furthermore, antibodies to the MUC1 peptide can block the activity of these CTL on MUC1$^+$ target cells (11–13). Thus, the murine immunogenicity studies become relevant for the design of methods of immunizing humans against cancer. With this in mind, we have examined the immunogenicity of various MUC1 antigens in mice: (*a*) whole MUC1$^+$ molecules expressed on murine 3T3 cells (but differently glycosylated to human MUC1); (*b*) human breast milk (HMFG); (*c*) synthetic peptide C-p13–32 of the VNTR of 20 amino acids; (*d*) a fusion protein containing 5 such repeats; and (*e*) synthetic peptides made from sequences NH_2-terminal and COOH-terminal to the VNTR. In brief, the tumor can totally immunize for a subsequent challenge and induce significant cellular but little humoral immunity; by contrast, the different soluble antigens and peptides induce significant humoral but less cellular immunity.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies to CD3 (KT3.2), CD4 (H129.19), CD8 (53–6.72), IFN-γ (R4–6A2), and $H2^d$ (34.12S) were prepared from either ascites or tissue culture supernatant (14–18). MUC1 antibodies were: VA1 and VA2, produced against a glutathione-*S*-transferase-MUC1 bacterial fusion protein which contains five VNTR repeats (10); BC2, made against HMFG which reacts with the peptide epitope APDTR from the VNTR repeat (19); BCP7–9, produced against MUC1 VNTR synthetic peptide (9); and 3E1.2, reactive with the carbohydrate epitope (Glycosyl Tn; Ref. 20). Other antibodies were CC5 (reactive with Le^a antigen) (21), anti-Ly2.1 (to mouse Ly2.1), and M3.1 (anti-MUC3 Mab).[4]

Synthetic Peptides, Fusion Protein, and HMFG Production and Immunization. Peptides C-p13–32 (MUC1 VNTR), p31–55, and p51–70 (NH_2-terminal to VNTR) and p344–364 and p408–423 (COOH-terminal to VNTR) were synthesized using an Applied Biosystems Model 430A automated peptide synthesizer (Refs. 22 and 23; sequences shown in Table 1). The mouse CD4 NH_2-terminal region peptide (T4N1) was also synthesized and used as a negative control peptide (Table 1). HMFG was isolated from human milk (19). A fusion protein containing five VNTR repeats coupled to GST was produced by subcloning the cDNA into the bacterial expression vector pGEX-3X (10; Table 1).

Female BALB/c mice (aged 8 weeks) were immunized i.p. with 50 μg of either fusion protein, HMFG, C-p13–32 (coupled to diphtheria-toxoid with glutaraldehyde), or T4N1 (coupled to diphtheria toxoid) emulsified in complete Freund's adjuvant, and this was repeated 4 and 6 weeks later in PBS. Prior to tumor injection and after tumor rejection, mice were bled, and the serum was tested by an ELISA for antibody production against the relevant immunogens.

Tumors. The BALB/c mouse fibroblast cell line 3T3 was transfected with the MUC1 cDNA transmembrane form with the *ras* gene, and a cell line MUC1$^+$3T3 was developed (obtained from Dr. D. Wreschner, Tel Aviv University, Israel; Refs. 24 and 25). Mice received a 0.2-ml s.c. injection of various tumor cell doses in PBS, and subsequent tumor growth measured. All

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[1] Supported by The Australian Cancer Foundation and Austin Research Institute.

[2] To whom all requests for reprints should be addressed.

[3] The abbreviations used are: MUC1, mucin 1; cDNA, complementary DNA; VNTR, variable number of tandem repeats; CTL, cytotoxic T lymphocytes; HMFG, human milk fat globule; IFN-γ, γ-interferon; GST, glutathione-*S*-transferase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; pagal, phenyl *N*-acetyl-α-D-galactosaminide; DTH, delayed-type hypersensitivity; IL, interleukin.

[4] V. Apostolopoulos, P-X. Xing, and I. F. C. McKenzie, unpublished data.

Table 1 *Sequences of synthetic peptides*

Peptide	Amino acid sequence
MUC1 VNTR	
Cp-13-32	C-PAHGVTSAPDTRPAPGSTAP
Fusion protein	(PAHGVTSAPDTRPAPGSTAP)x5-GST
NH_2-terminal region to MUC1	
p31-35	TGSGHASSTPGGEKETSATQRSSVP
p51-70	RSSVPSSTEKNAVSMTSSVL
COOH-terminal region to MUC1	
p344-364	NSSLEDPSTDVVQELQRDISE
p408-423	TGFNQYKTEAASRVNL
Mouse CD4	
T4N1	KTLVLGKEQESAELPCEY

measurements were performed with dial gauge calipers (Schnelltaster; H. C. Kroplin, Hessen, Germany), and the size of the tumors were expressed by the area of the tumor size (cm^2; diameter x diameter). The murine DBA/2 mastocytoma cell lines P815 and MUC1$^+$P815 (containing the cDNA of the transmembrane form of MUC1) were obtained from Dr. B. Acres (Transgene, Strasbourg, France; Ref. 26).

MUC1$^+$3T3 tumor injections were given s.c. in 0.2 ml containing the appropriate tumor dose. Mice treated with CD3, CD4, CD8, and IFN-γ antibodies were given three i.p. injections of 0.2 ml on days -2, 0, and $+2$ and injected s.c. with the tumor on day 0. In some experiments (see below) on day 5 (tumor size,~ 0.15 cm^2), mice were also treated with rabbit complement (fresh serum, 0.2 ml i.v.) and anti-MUC1 (BC2) antibody (0.2 ml i.p.) on days 5 and 7.

Serology. For flow cytometry, antibodies (250 μl) as supernatant (Table 2) were added to 2×10^5 tumor cells and incubated for 1 h at 4°C. After washing with 0.5 ml (2% newborn calf serum/phosphate buffer), 100 μl 1:50 dilution of fluorescein isothiocyanate-conjugated sheep $(Fab')_2$ anti-mouse immunoglobulin (conjugate; Silenus, Melbourne, Australia) was added and incubated for 45 min at 4°C; after further washing, the cells were analyzed by flow cytometry.

The ELISA test was performed (19) such that 20 μg/ml of peptides or fusion protein or 10 μg/ml of HMFG were coated in the wells of a microtiter plate, nonspecific binding was blocked with 2% bovine serum albumin, and 50 μl of antibody were added for 2 h at room temperature. After washing, sheep anti-mouse immunoglobulin conjugated to horse radish peroxidase conjugate (Amersham, Buckinghamshire, United Kingdom) was added and incubated at room temperature; the test was developed using 50 μl, 0.03% 2,2′-azino-di(3-ethylbenzthiazoline sulfonate) (Amersham), 0.02% H_2O_2 (100 volumes; Ajax Chemical) in 0.1 M citrate buffer (pH 4.0), incubating for 10–15 min at room temperature until the desired intensity was achieved. The absorbance was read at 405 nm in a plate reader.

***In Vivo* Depletion of Cells.** To ensure that the antibodies to CD3, CD4, and CD8 were depleting or blocking CD3$^+$, CD4$^+$ and CD8$^+$ T-cells, a serological analysis of residual cells was performed using the antibodies to CD3, CD4, and CD8. Briefly, spleen and lymph node cells were removed from normal or antibody-treated BALB/c mice. The lymphocytes were teased out, washed in Dulbecco's modified Eagle's medium, and incubated at 37°C for 5 min in 0.83% ammonium chloride to lyse RBC. Serology tests were performed where 2×10^5 spleen/lymph node cells from mice were added to a 1:500 dilution of anti-CD3, anti-CD4, and anti-CD8 ascites. Following extensive washing, the cells were incubated with (mouse thymus cell-absorbed) rat anti-mouse IgG and incubated for 30 min on ice. The cells of the antibody-treated mice were tested, and it was found that the CD3$^+$ cells were depleted and CD4$^+$ and CD8$^+$ cells had been blocked (data not shown; Refs. 14–16, 27, 28).

CTL Assay. BALB/c mice were injected i.p. with 5×10^6 MUC1$^+$3T3 cells. After 14 days, they were given a second i.p. inoculation and were sacrificed 1 week later; then the peritoneal exudate cells were collected and washed in 2% fetal calf serum/PBS. For mice immunized with peptide, fusion protein and HMFG spleen cells were collected before being challenged with the tumor. The target cells, P815, and MUC1$^+$P815 cells were either not treated or treated with 5 mM pagal for 48–72 h (to inhibit *O*-linked glycosylation; Sigma Chemical Co., St. Louis, MO) prior to use in a standard ^{51}Cr release assay (29). Tumor cells (10^6 cells) were radiolabeled with 100 μCi of $Na_2{}^{51}CrO4$ (Amersham Corp., Arlington Heights, IL) for 1 h at 37°C, followed by extensive washing. Peritoneal exudate cells and target cells were resuspended in culture medium (RPMI) and then combined at various effector:target ratios in 96-well, U-bottomed plates. The plates were then centrifuged at $100 \times g$ for 3 min and incubated for 4 h at 37°C with 5% CO_2, when the supernatants were collected and radioactivity was quantitated in a gamma counter. The spontaneous release of ^{51}Cr was determined by incubation of the target cells alone, while maximum release of ^{51}Cr was determined by treatment with 10% sodium dodecyl sulfate; the percentage of specific release was determined as:

$$\% \text{ specific release} = \frac{\text{Experimental} - \text{spontaneous}}{\text{Maximum} - \text{spontaneous}} \times 100$$

Antibody-blocking assays were performed by incubating the effector and target cells at a constant 50:1 ratio with 1:100–1:2000 dilutions of anti-CD4, anti-CD8 (both of IgG2a phenotype) ascites and an irrelevant IgG2a antibody (to MUC3); the CD8 antibody used has been shown to block CTL responses, whereas the CD4 antibody cannot (15, 27).

Induction of DTH. To induce DTH in mice, cyclophosphamide (Farmitalia Carlo Erba, Victoria, Australia) at a dosage of 200 mg/kg body weight was injected into the peritoneal cavity 2 days before an i.p. injection of either 5×10^6 MUC1$^+$3T3 cells, 50 μg of C-p13–32 (coupled to diphtheria toxoid), fusion protein, or HMFG. Six days later, the hind footpads were injected (20 μl) with either 10^5 3T3 or MUC1$^+$3T3 (freeze/thawed five times), 50 μg of HMFG, 50 μg fusion protein, or 50 μg of C-p13–32 (coupled to keyhole-limpet hemocyanin using glutaraldehyde) or an equivalent volume of PBS. The DTH response was measured at 48 h later by measuring the width and the

Table 2 *Serological analysis of MUC1$^+$ 3T3 cells (expressed as % positive cells)*

			3T3		MUC1$^+$3T3		P815		MUC1$^+$P815		T47D[b]		MCF7	
Antibody	Immunogen[a]	Epitope	−pagal	+pagal	−pagal	+pagal	−pagal	+pagal	−pagal	+pagal	−pagal	+pagal	−pagal	+pagal
3E1.2	Tumor tissue	glycolyl-Tn	4.7	0.19	93.0	30.9	10.7	3.1	95.9	16.1	92.6	47.8	94.7	50.7
CC5	HMFG	Le^a	2.3	2.8	4.3	1.5	4.9	4.6	3.8	8.8	42.5	10.9	19.9	9.1
BC2	HMFG	APDTR	3.2	7.1	99.0	96.9	11.8	6.3	94.3	88.7	96.9	92.2	78.2	87.4
VA1	Fusion protein	RPAPGS	15.1	11.24	74.1	88.9	19.6	2.2	28.1	86.4	78.9	92.1	77.1	91.1
VA2	Fusion protein	DTRPA	0.6	1.48	98.7	90.4	10.9	3.8	78.8	95.3	98.1	94.4	92.3	84.9
BCP7	Peptide	VTSA	2.7	1.9	57.4	89.4	10.8	2.7	7.7	96.4	52.2	81.2	29.3	85.3
BCP8	Peptide	DTR	1.7	1.5	99.7	96.9	10.4	3.2	85.5	97.0	98.3	95.9	80.2	90.9
BCP9	Peptide	GSTAP	1.4	1.4	59.3	73.4	7.8	2.9	19.4	88.9	44.2	64.2	27.8	76.0
BCP10	Peptide	RPAP	3.9	0.7	26.0	63.3	11.3	10.8	5.7	76.3	12.6	80.5	20.4	71.6
anti-H2[d]			84.9	90.4	92.1	80.9	81.9	94.6	92.8	89.9	7.3	5.1	3.9	3.9
anti-Ly2.1			0.9	0.58	0.7	2.2	6.0	3.9	4.8	5.5	5.2	5.9	6.3	9.7

[a] Peptide sequence, PDTRPAPGSTAPPAHGVTSAPDTR; fusion protein sequence; (PDTRPAPGSTAPPAHGVTSAPDTR)x5.
[b] T47D and MCF7, breast cancer cell lines.

thickness of the footpad and calculating their product. All measurements of footpads were performed with dial gauge calipers (Schnelltaster; H. C. Kroplin). The phenotype of the DTH cells was determined by treating mice with CD4 and CD8 antibodies 4 and 2 days prior to immunization.

RESULTS

Serological Analysis of MUC1$^+$3T3 Cells

In vitro MUC1$^+$3T3 cells did not appear to be different from normal 3T3 cells because they had the same appearance and growth characteristics (data not shown). By serological analysis, MUC1$^+$3T3 cells expressed high concentrations of MUC1 and were of the H-2^d phenotype. When a more comprehensive typing was performed (Table 2), it was clear that antibodies to MUC1 VNTR peptides reacted with MUC1$^+$3T3 and MUC1$^+$P815 similarly to the human breast cancer cell lines T47D and MCF7 (typing with: anti-HMFG, BC2 antibody; anti-fusion protein antibodies; VA1 and VA2; and anti-MUC1 peptide antibodies, BCP7, BCP8, BCP9 and BCP10). However, murine tumors were differently glycosylated than the human tumors as MUC1$^+$3T3 and MUC1$^+$P815 cells were reactive with anti-carbohydrate (3E1.2) antibody (epitope, glycolylsialyl-Tn) but not with other antibodies to carbohydrate (CC5 epitope, blood group Le^a). This shows that the protein antigens are intact but the glycosylation is altered, hardly surprising as mice and humans have different glycosyl transferases and therefore different patterns of glycosylation. However, after removal of sugars by pagal treatment, the antibodies to MUC1 VNTR (non-APDTR reactive antibodies, VA1, BCP7, BCP9, and BCP10), which previously had weak or no reaction with cell lines, became reactive as their epitopes were now exposed. However, there was no difference noted with the (AP)DTR(PA) reactive antibodies, but there was a major difference in reactivity with the carbohydrate reactive antibody (3E1.2) where the staining became weak or negative after pagal treatment, indicating that the pagal was indeed removing *O*-linked sugars because the epitope of 3E1.2 is *O*-linked to the protein core of the mucin (30). The typing was repeated at different times, and the same results were obtained, indicating that the phenotype was stable (data not shown).

In Vivo Growth of MUC1$^+$3T3 Cells

BALB/c mice received a s.c. injection of 5×10^6 MUC1$^+$3T3 or 3T3 cells, and the subsequent growth measured; 3T3 cells grew progressively (Fig. 1*A*) and were not rejected, as would be expected in BALB/c mice. By contrast, the MUC1$^+$3T3 cells grew progressively until day 10, when they started to shrink and had gradually disappeared by day 18 (Fig. 1*A*). Thus, the human MUC1$^+$ gene product appears to confer an immunogenicity on 3T3 cells, leading to their rejection. This was indeed the case because the subsequent challenge with 5×10^6 MUC1$^+$3T3 or 3T3 cells demonstrated the total resistance in immunized mice to the growth of MUC1$^+$3T3 cells, whereas 3T3 cells grew, *i.e.*, the immunogenicity was found only in MUC1$^+$-bearing tumors and was specific for this antigen. Specificity and memory indicate an immune response to MUC1$^+$ and not some other effects such as MUC1$^+$ effecting the growth properties of 3T3. After 8 weeks of repeated experiments using tumors passaged *in vivo*, we noted that not all of the mice rejected their tumors, and up to 30% of MUC1$^+$ tumors continued to grow. When these tumors were excised and MUC1$^+$ was measured serologically, a proportion of cells in the tumors were MUC1$^-$ *i.e.*, some of the MUC1$^+$-transfected cells had lost their capacity to express MUC1$^+$ *in vivo* (we did not determine whether the genes were still present). Such observations have been reported elsewhere with rat tumors (31), presumably due to unstable expression of MUC1. In all our subsequent studies, we ensured that tumors were 100% MUC1$^+$ when used by serologically testing the MUC1 expression with the anti-HMFG antibody BC2.

Dose-response studies were also performed to find a dose of tumor cells which could grow in BALB/c mice. Groups of 10 BALB/c female mice were injected with various doses of MUC1$^+$3T3 tumor cells, and their growth was subsequently measured. In mice injected with doses of 5×10^7 (the highest dose feasibly given), 1×10^7, 5×10^6, 1×10^6, or 5×10^5 cells, tumors grew, and by day 18, they had all been rejected, *i.e.*, no dose could be found where tumor growth overcame the immune response. By contrast, in mice injected with doses of 1×10^5 or less, no tumors were observed (Fig. 1*B*). For subsequent experiments, either a "high dose" (5×10^6) or "low dose" (1×10^6) of cells was used.

T-Cell Immune Responses to MUC1$^+$3T3 Cells

Cellular immunity was most likely to be the mode of rejection because it is the commonest mode of rejecting tumor allografts in mice; this was confirmed by the ability of anti-CD3 antibodies to totally abrogate immunity (Fig. 2). To determine whether CD4$^+$ or CD8$^+$ cells were responsible for rejection, mice received multiple doses of anti-CD4 or anti-CD8 antibody because these were known to cause immunosuppression in other models (15, 27). Functional CD4 cell depletion by blocking had a transient effect on tumor growth, and tumors were rejected in a similar fashion to untreated mice (Fig. 2). By contrast, anti-CD8 treatment led to prolonged tumor growth. We conclude that CD3$^+$ cells are totally responsible for rejection, CD4$^+$ cells have a minimal effect, and CD8$^+$ cells are the major effectors of graft rejection (Fig. 2). It was noted that in anti-CD8-treated mice, the tumors were smaller than those receiving anti-CD3 (Fig. 2); clearly, the anti-CD8 antibody was not as effective as total T-cell removal

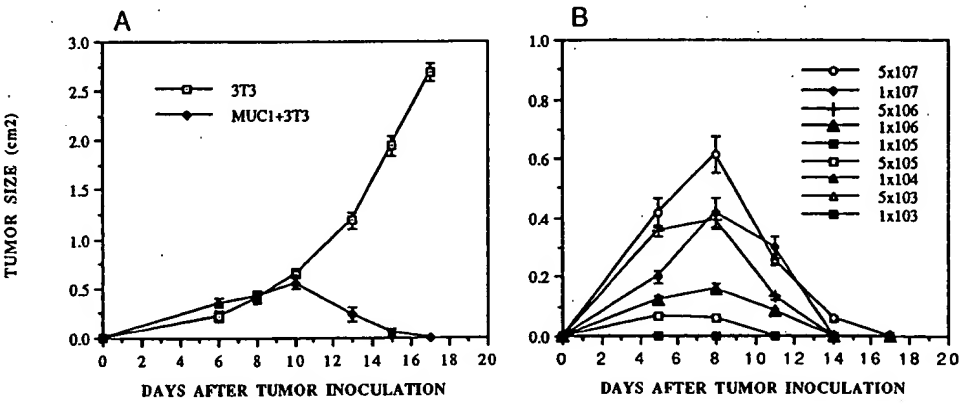

Fig. 1. *A*, growth of 5×10^6 3T3 and MUC1$^+$3T3 cells in BALB/c mice showing tumor size and time after tumor inoculation. *B*, dose-response of MUC1$^+$3T3 cells in BALB/c mice for s.c. tumors. Doses range from 10^3-5×10^7 cells. *Ordinate*, tumor size (cm^2); *abscissa*, days after tumor inoculation. There was no growth at the lowest three dose levels, and the symbols are superimposed.

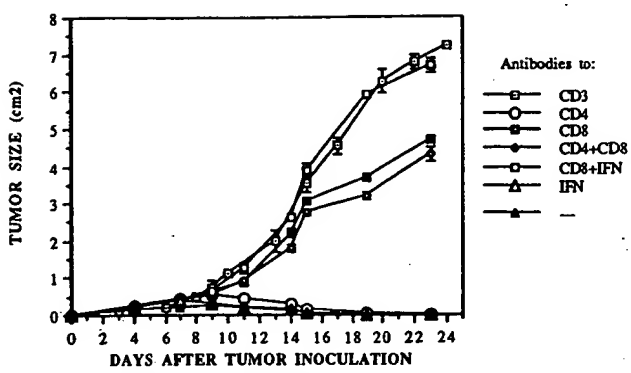

Fig. 2. All BALB/c mice treated with anti-CD3, anti-CD4, anti-CD8, and anti-IFN-γ on −2, 0, +2 days and challenged with 5×10^6 MUC1$^+$3T3 cells. *Ordinate,* tumor size (cm^2); *abscissa,* days after tumor inoculation.

with anti-CD3 antibody. CD4 cells having a minor effect was unlikely because the combined action of anti-CD4 and anti-CD8 was no better than anti-CD8 alone. However, we noted that anti-IFN-γ treatment (of no effect used alone) combined with anti-CD8 gave a similar effect with anti-CD3 (Fig. 2); thus, IFN-γ plays a role in tumor graft rejection, which is mediated by CD8$^+$ cells and IFN-γ.

Thus, MUC1$^+$3T3 cells could immunize BALB/c mice against MUC1 carried on the 3T3 cells and gave rise to cellular immunity expressed by CD3$^+$8$^+$ cells but not by CD3$^+$4$^+$ cells; there was little humoral immunity because no anti-MUC1 antibodies were found (see below; Fig. 7). To measure the various parameters of the immune response, we examined DTH and CTLs.

DTH. Clearly, the immune response was cellular and due to CD8$^+$ cells. To determine whether this also involved a DTH response (usually considered to be mediated by CD4$^+$ cells) or a CTL response (usually CD8$^+$), mice were immunized with MUC1$^+$3T3 cells, and a DTH was performed by injecting the hind footpads with various antigens (Fig. 3). Preliminary studies demonstrated that, in the absence of cyclophosphamide, no measurable DTH responses occurred. A DTH response was detected in the footpads injected with killed (freeze/thawed five times) MUC1$^+$3T3 cells and a weaker response when challenged with either HMFG, fusion protein-GST, or Cp13–32-keyhole-limpet hemocyanin (Fig. 3). These responses were clearly specific because 3T3 cells elicited no response. To determine whether the DTH response was mediated by CD4$^+$ or CD8$^+$ cells, mice were injected with anti-CD4 and anti-CD8 antibodies, and the DTH response measured. Anti-CD4 totally blocked DTH reactions, while anti-CD8 partially blocked DTH reactions but to a lesser extent when challenged with MUC1$^+$3T3 cells, Cp13–32, HMFG, and fusion protein (Fig. 3). Thus, the cells which cause the effective immune response to human MUC1-CD8$^+$ T cells were not the same as those eliciting a DTH response, although CD8$^+$ cells certainly contributed to the DTH.

CTLs. Cytotoxic assays were performed. After MUC1$^+$3T3 cell immunization, there was ~60% lysis of MUC1$^+$P815 targets treated with pagal; untreated MUC1$^+$P815 targets and nontransfected P815 targets were not lysed (Fig. 4). Pagal treated and nontreated 3T3 and MUC1$^+$3T3 targets gave no lysis because they are poor targets for CTL assays. To determine the phenotype of the CTLs, anti-CD4 and anti-CD8 antibodies were used in blocking studies; the anti-CD8 reagent (53–6.7) was known to be capable of blocking T-cell lysis by CD8$^+$ cells. This proved to be the case in these studies because anti-CD8 could totally block CTL reactions at 1:1000 antibody dilution, whereas anti-CD4 and anti-MUC3 (control antibody) were without effect (data not shown). Since only CTLs were found to pagal-treated MUC1$^+$P815 targets and since non-APDTR reactive anti-MUC1 antibodies (VA1, BCP7, BCP9, and BCP10) became reactive with pagal-treated MUC1$^+$3T3, MUC1$^+$P815, T47D, and MCF7 cells (Table 2), it is clear that these antibody-reactive and T-cell reactive epitopes are hidden and then exposed after pagal treatment.

Thus, mice resistant to MUC1$^+$3T3 cells have CD8$^+$ T-cell immunity, CD4$^+$ DTH, a detectable CD8$^+$ CTL response, and little or no antibody (see below). Because the CTL response (at least at the level of the T-cell phenotype) correlated with the effector cell phenotype in rejecting tumors, it would appear to be the more appropriate response to measure.

Immune Responses: B-Cells

While it was shown above that cellular immunity was effective and little antibody was made, the role of antibody was not clear. Furthermore, mice generally make poor antibodies and mobilize complement so poorly that they are not the species of choice in which to study antibody-mediated destruction of grafts, unless certain conditions are met: (*a*) the provision of sufficient antibody (be it polyclonal or monoclonal); (*b*) the provision of sufficient complement; and (*c*) high density of surface antigens. The MUC1$^+$ antigen density is high; therefore, additional antibody and complement were provided. In addition, the mice were immunosuppressed with CD3 to remove any component of cellular immunity (14). In spite of large amounts of antibody and complement (as described in "Materials and Methods") capable of rejecting skin allograft and xenografts, the tumors grew progressively, indeed, at the same rate as in mice not receiving antibody (Fig. 5). Thus, antibody and complement are unable to cause rejection of MUC1$^+$3T3 cells.

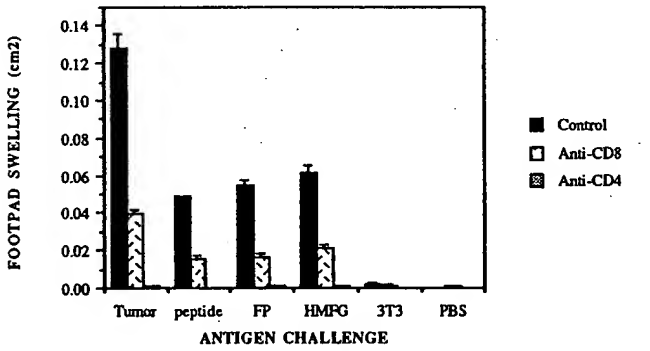

Fig. 3. DTH response measured at 48 h in mice immunized with MUC1$^+$3T3 cells and challenged with dead 3T3 and MUC1$^+$3T3 cells, Cp13–32, fusion protein, HMFG, and PBS in their hind footpads. Control, (■); mice treated with anti-CD4, (▨); and mice treated with anti-CD8 (▽). Footpad swelling was measured by measuring the width by the thickness of the footpad and calculating their product (cm^2).

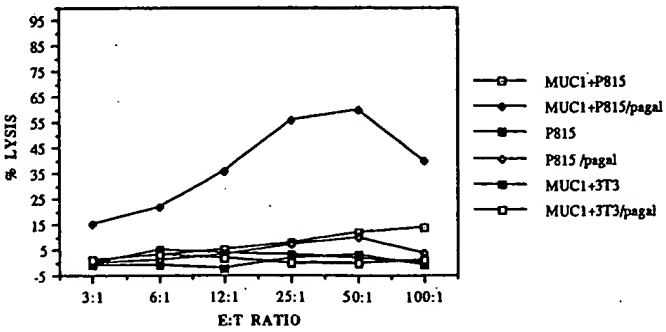

Fig. 4. CTL assay using different targets as shown in key.

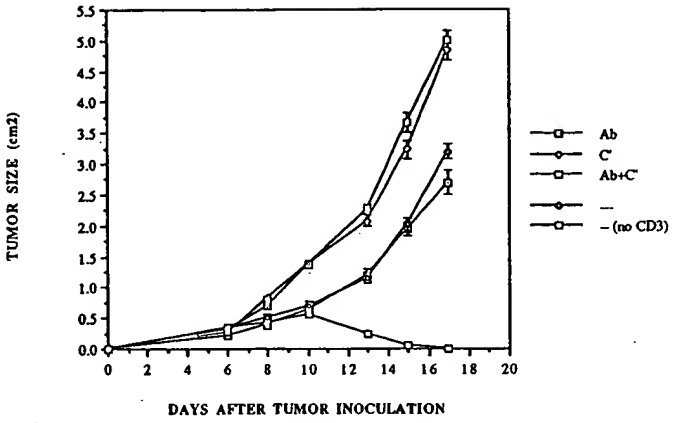

Fig. 5. *In vivo* resistance to antibody (*Ab*) and complement (*C'*). BALB/c mice were injected with 5×10^6 MUC1$^+$3T3 cells on day 0. On, days −2, 0, and +2, mice were treated with anti-CD3 antibody; on days 5 and 7, mice were treated with either antibody, complement, antibody and complement, or PBS. *Ordinate*, tumor size (cm^2); *abscissa*, days after tumor inoculation.

Immunization with HMFG, Peptides, and Fusion Protein

The preceding defines a model of the murine immune response to human MUC1 transfected into 3T3 cells and forms the basis for using various immunogens to generate a similar or greater immune response with synthetic materials as that produced with cellular MUC1. The aim was clearly to substantially decrease tumor growth after immunization. As immunogens, natural mucin (HMFG), synthetic products (MUC1 peptides made of VNTR dimers), and a 5xVNTR repeat fusion protein were used. It should be noted that without prior immunization, tumors are rejected after 18 days, such mice then being resistant to a subsequent challenge. Thus, there is a "window" of ~18 days when tumors will be rejected, and immunization could lead either to tumors not appearing or being of reduced size during this time.

To examine the immunogenicity of HMFG, fusion protein, and synthetic peptides, groups of 15 BALB/c mice were immunized with 50 μg of these materials and challenged with 1–5 $\times$ 10^6 3T3 or MUC1$^+$3T3 cells. The 3T3 cells had the same progressive growth in all immunized and nonimmunized mice; therefore, there were no nonspecific effects of the immunization procedures. When mice were challenged with the lower dose of 1×10^6 cells, significant differences were noted as compared to the nonimmunized control (Fig. 6*A*). Thus, on day 6, mice immunized with either the peptide or fusion protein had tumors ~25% that of controls; immunizing with HMFG was less effective, tumors being ~60% the size of controls. However, when challenged with 5×10^6 MUC1$^+$3T3 cells, there was some difference in tumor size compared to the controls (Fig. 6*B*), but this difference was not as obvious by challenging with a lower dose. As expected with subsequent tumor challenge, the peptide-immunized mice which had rejected the tumor were now resistant to tumor challenge (Fig. 6*C*). Thus, immunizing mice with peptides, fusion proteins, or HMFG and challenging with a low dose of MUC1$^+$3T3 cells gave rise to some antitumor effect. Although the VNTR-containing peptide, fusion protein, and HMFG gave some degree of protection, mice immunized with the NH_2 and COOH terminal peptides of MUC1 had no significant protection (Fig. 6*D*), indicating that these peptides do not induce immunity to MUC1 and also showing that the immunization procedure itself was without effect. To measure the various parameters of the immune response, we examined MUC1 antibody production, DTH, and CTLs.

Antibody. Mice immunized with peptides, fusion protein, or HMFG had high levels of anti-MUC1 antibody (Fig. 7) both before and after tumor injection. Thus, immunization gave rise to high levels of antibody but apparently little cellular immunity, as shown by the minor effect on the tumors. It was of interest that mice immunized with the control peptide (T4N1) and which had rejected the tumor did not produce antibodies against MUC1; nor did the mice immunized with peptide and other immunogens have an increase in antibody titer after rejecting the tumor (Fig. 7).

DTH. Mice immunized with HMFG, Cp13–32, and fusion protein-GST had DTH responses to the various MUC1 antigens (Fig. 8), which could be inhibited by CD4 (totally) and CD8 (partially) antibodies (Fig. 8) Thus, immunization with the three agents gave rise to some degree of cellular immunity but not sufficient to greatly inhibit tumor growth.

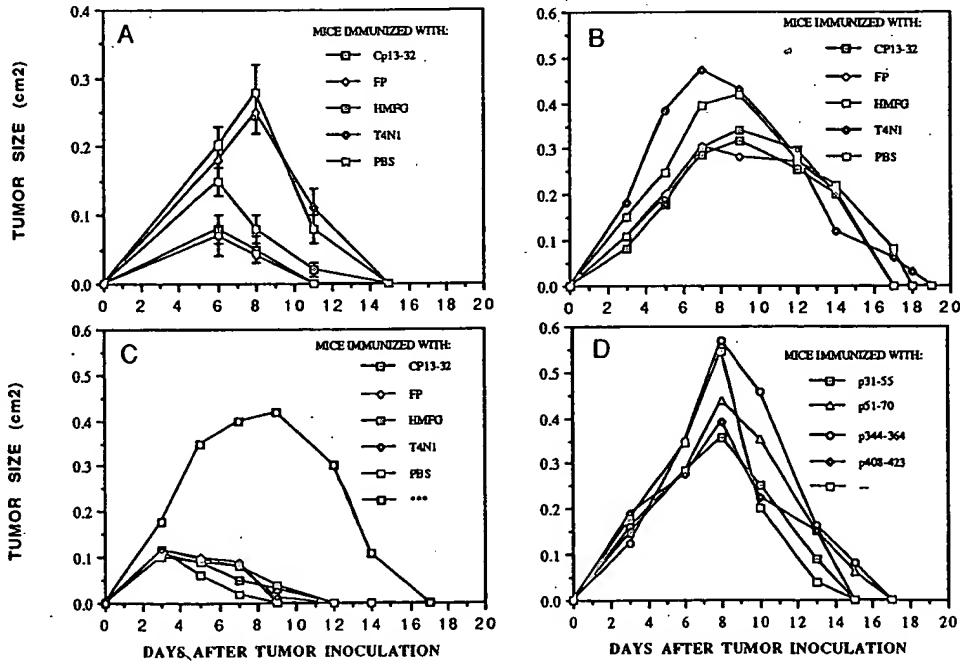

Fig. 6. Mice previously immunized with (50 μg) fusion protein, HMFG, T4N1, or Cp13–32 and a control group were injected with tumors on day 0. *A*, 1×10^6 MUC1$^+$3T3 cells; *B*, 5×10^6 MUC1$^+$3T3 cells; *C*, after rejection of all tumors, mice were rechallenged with 5×10^6 MUC1$^+$3T3 cells. ***, nonimmune control mice. *D*, 5×10^6 MUC1$^+$3T3 cells in mice immunized with (50 μg) NH_2-terminal to VNTR peptides (p31–55 and p51–70) or with COOH-terminal peptides to the VNTR (p344–364 and p408-p423). —, nonimmune control group. *Ordinate*, tumor size (cm^2); *abscissa*, days after tumor inoculation.

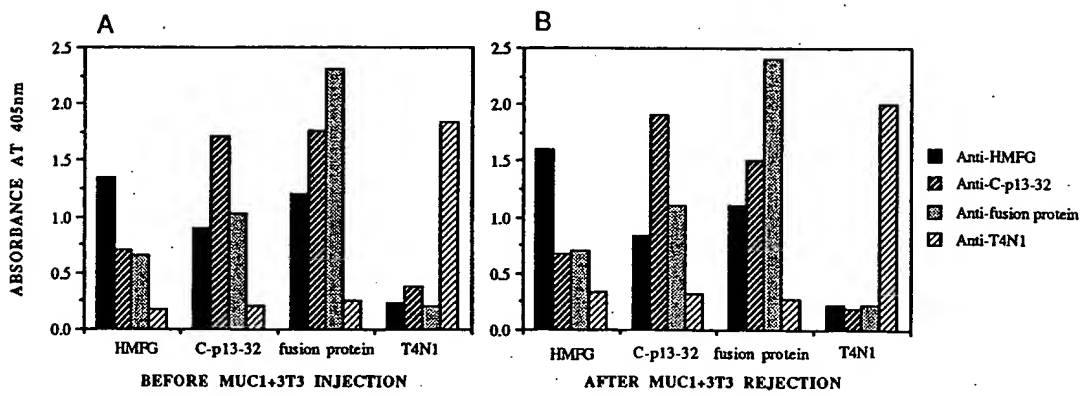

Fig. 7. Mice immunized with (50 μg) fusion protein, HMFG, T4N1, Cp13–32, or PBS. *A*, mice were bled, and sera were tested by ELISA for anti-MUC1 antibodies prior to MUC1$^+$3T3 cell challenge. *B*, mice were bled, and sera were tested for anti-MUC1 antibodies after tumor rejection.

CTL Assay. CTL assays were performed from spleen and lymph node cells of immunized mice, and no cytotoxic cells were detected. Thus, the various immunization procedures appeared to bias the immune response to antibody production rather than cellular immunity. Table 3 summarizes the differences in immunizations with cellular and synthetic antigens.

DISCUSSION

Recent studies of MUC1 indicate that murine studies of immunogenicity could be applicable to patients. This conclusion was based on the observation that: (*a*) mice recognize human MUC1 in cancer cells and HMFG, detecting either carbohydrate or peptide epitopes [Refs. 9, 10, and 20; we will concentrate here on peptides (carbohydrates are the focus of other studies, Ref. 32)]; (*b*) the predominant peptide detected by mice is the amino acid sequence APDTR (5, 9, 10); and (*c*) humans also recognize MUC1 by a cytotoxic immune response, which can be blocked by anti-MUC1 antibodies (to APDTR) and is, therefore, presumably to the same or adjacent sequence (11–13). Indeed, such studies have now been performed in different laboratories in breast cancer (11, 12), pancreatic cancer (11, 12), and recently in ovarian cancer (13). Thus, studies of the immune response (and its manipulation) to the sequence APDTR in the mouse may well be applicable to the human response to MUC1, particularly in breast, pancreatic, and ovarian cancer. With this in mind, we established a model using human MUC1 expressed after transfection in murine 3T3 cells. In this model, MUC1 cellular molecules are highly immunogenic, leading to resistance after challenge with 5×10^7 cells (Fig. 1*B*); furthermore, immunizing with as few as 10^3 MUC1$^+$ cells renders mice resistant to further challenge (data not shown). The immune response induced by these tumors is of interest because it is predominantly cellular in type with little or no antibody produced. Tumor rejection is mediated by CD3$^+$ cells with a CD8$^+$ phenotype; CD4$^+$ cells play little role in the response, although IFN-γ is involved. There are CD8$^+$ CTL present which presumably mediate rejection because they are of the same phenotype as the tumor rejecting cells; CD4$^+$(CD8$^\pm$) DTH cells are also present (Table 3). Because the CTL are CD8$^+$, it is likely that CTLs mediate rejection, either directly and/or with some role of IFN-γ. Thus, the response is of the TH_1 type, although mediated by CD8$^+$ and not by CD4$^+$ cells. Thus, efforts of immunizing to inhibit tumors should be to produce this phenotype, CD8 CTL cells and little antibody; the DTH response in this model seems to be irrelevant. The CD8$^+$ phenotype of the rejecting cells is not unique; we and others (33–35) have found such cells in rejecting skin grafts with a class I difference (class II generates CD4$^+$ rejecting cells) and in tumor models where CTL are required to inhibit tumor growth. We note that whole glycosylated tumors induce the CTL response, which we argue causes the tumors to disappear. However, such tumors *in vitro* are resistant unless deglycosylated; at present, we have no explanation of this paradox.

Against this background, mice were immunized with various agents to produce more rapid tumor rejection. We immunized mice with various MUC1 synthetic peptides containing either: (*a*) the APDTR from the VNTR; (*b*) peptides to the NH_2-and COOH-terminal portions of extracellular MUC 1; (*c*) a fusion protein containing five VNTR repeats-GST; or (*e*) HMFG. In contrast to immunization with the tumor, all these agents produced high antibody titres and a degree of cellular immunity but certainly not the level of immunity produced

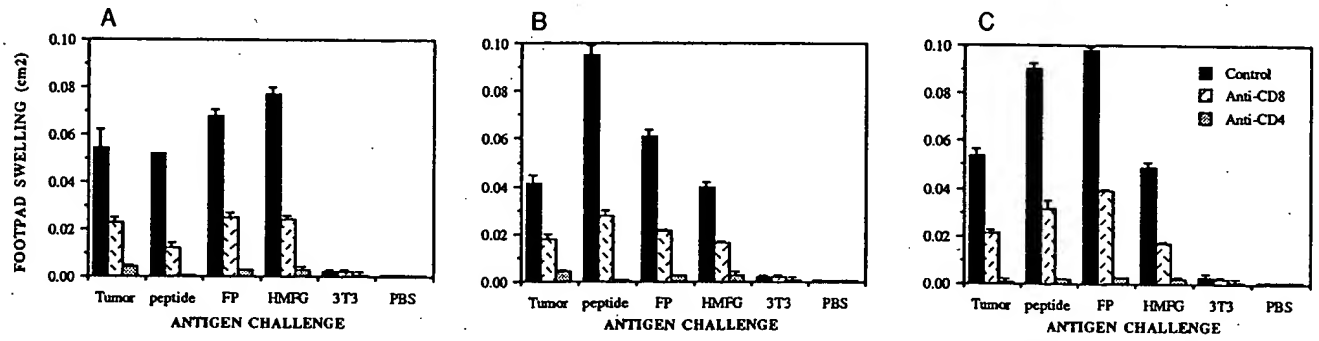

Fig. 8. DTH response measured at 48 h in mice immunized with (*A*) HMFG, (*B*) Cp13–32, and (*C*) fusion protein and challenged with dead 3T3 and MUC1$^+$3T3 cells, Cp13–32, fusion protein, HMFG, and PBS in their hind footpads. Control, ■; mice treated with anti-CD4 (▤); and mice treated with anti-CD8 (▽). Footpad swelling was measured by measuring the width by the thickness of the footpad and calculating their product (cm^2).

Table 3 *Differences in immunizations with cellular and synthetic antigens*[a]

Immunogens	Tumor rejection	Antibody	DTH	CTL
Tumor (MUC1$^+$ 3T3)	+++	+	+++	+++
Peptide	+	+++	+++	−
Fusion protein	+	+++	+++	−
HMFG (mucin)	+	+++	+++	−

[a] +++, high; +, low; −, absent.

by MUC1$^+$3T3 cells. When challenging with 10^6 cells, some antitumor immunity was apparent, but no CTLs could be detected, although measurable DTH responses could be elicited (summarized in Table 3); it is not clear whether this small response is due to nonmeasurable CTL. From these studies, it appears that: (*a*) antibody responses are neither useful nor desirable because they have little influence on the rejection procedure. Their presence is most likely to indicate the bias of the immune response in the humoral direction rather than to cellular immunity, *i.e.*, towards CD4$^+$ TH_2 cells; (*b*) DTH responses also do not seem to be entirely relevant. In the model, DTH responses are predominantly mediated by CD4$^+$ cells and can be induced by tumor cell rejection but also by HMFG, fusion protein, and peptide under circumstances where little antitumor effects were noted; (*c*) CTL responses (TH_1 type) appeared to most closely indicate the underlying cell type required for immunity, and their induction should be the aim of subsequent immunizing protocols; and (*d*) HMFG, fusion protein, and peptide immunization in the current form are not ideal and did not give rise to the desired type of immunity in that the peptides gave rise to antibody responses and no measurable cytotoxic response. It has been suggested that peptide immunization does not lead to CTL responses (36–38), although immunization with HIV and herpes simplex virus peptides give CD8$^+$ CTL (39, 40). Indeed, by modifying the fusion protein, we have been able to induce CD8$^+$ cells.[5] It should be noted that the tumors were administered s.c., whereas the immunizing agents were given i.p. Could this explain the difference in immunity? Probably not because when MUC1$^+$ 3T3 cells are given i.p., the same CTL response is induced (data not shown).

All of the immunizing moieties used in this study gave rise to significant antibody titers, but with the tumor used, antibody-mediated immunity appears to be of little relevance (Fig. 5). However, we note that mice are not good experimental models to examine the effects of the antibody and complement; their endogenous complement system is poorly activated in such studies. With this in mind, we added additional complement (from rabbits) to mice with MUC1 tumors; there was no effect noted. Thus, in mice with this tumor, antibody appears to be irrelevant. However, the very presence of large amounts of antibody indicates that little cellular immunity is likely because these features are usually the reciprocal of each other (Table 3). [In classical studies, mice immunized with flagellin make good antibody and poor cellular immunity; acetoacetylated flagellin does the reverse (41)]. In this light, studies are now in progress to alter the form of the peptides by chemical modification and to alter the type of immune response by the judicious use of cytokines and anti-cytokine antibodies. [Our findings with anti-IFN-γ would indicate that perhaps TH_1 cells make some IFN-γ, which has an antitumor effect (Fig. 2)].

With these conclusions, we can now review other studies in animals using MUC1. The whole *MUC1* gene was transfected into the mouse mammary epithelial tumor cell 410.4, and after immunization with these cells, there was a reduction in tumor incidence after challenging with 10^5 MUC1$^+$410.4 cells and a delay in tumor growth after challenging with 10^6 cells (it should be noted that tumors were still growing after 56 days; Ref. 42). However, in our model, immunization with tumor totally inhibited subsequent tumor challenge; thus, the tumor itself is highly immunogenic. The MUC1$^+$3T3 tumor we have appears to be different from other MUC1$^+$ tumors in that it is rapidly rejected by all mice and gives rise to good cellular immunity, mediated by CD3$^+$ CD8$^+$ cells; CD4$^+$ cells do not appear to be involved in the rejection process. In a separate study (38), mice immunized with a synthetic peptide derived from the 20- amino acid sequence of the MUC1 VNTR gave strong DTH reactions, measured using synthetic peptides containing the PDTRP sequence. DTH responses were also elicited with synthetic MUC1 peptides (containing PDTRP) following immunization with MUC1 transfected into a mouse mammary tumor. Immunizing with synthetic MUC1 peptides also partly inhibited the growth of MUC1-transfected tumor cells and prolonged the survival of tumor-bearing mice (36). In an additional study, vaccinia virus recombinants expressing the transmembrane (T) and secreted (S) form of MUC1 (T-MUC1-VV and S-MUC1-VV, respectively; Ref. 31) were used to immunize rats; those immunized with the T-MUC1-VV construct showed high antibody titers and inhibited the growth of 82% of T-MUC1$^+$ tumors and 61% of S-MUC1$^+$ tumors; the S-MUC1-VV recombinant protein was a less effective immunogen because vaccination protected only 30% of rats challenged with T-MUC1$^+$ and S-MUC1$^+$ tumors. It was suggested that, as there were high antibody titers in rats immunized with T-MUC1-VV, then antibodies are involved in this model, which contrasts with our findings. Recently, it was shown by others[6] that a proportion of DBA/2 mice immunized with vaccinia virus-MUC1 were protected from growth of P815-MUC1 tumors when challenged. Immunized mice had high titers of IgG antibodies, but this was not considered to be the cause of subsequent tumor rejection; no specific CTL were found in these studies.

In none of these models were anti-MUC1 CTL detected; indeed, the description here is the first to describe murine anti-MUC1 CTL. The CTL studies were of interest with regard to the APDTR epitope, which is the site of action of most anti-MUC1 antibodies which react with the core protein of MUC1. Our studies suggest that APDTR is possibly not the epitope reacting with the CTL because antibodies reacting with APDTR (BC2, VA2, and BCP8, which react with DTR) did not have any difference in reactivity after deglycosylation, *i.e.*, the APDTR epitope was not blocked for these antibodies. By contrast, non-APDTR antibodies (VA1, RPAPGS; BCP7, VTSA; BCP9, GSTAP; and BCP10, RPAP) all developed antibody-reactive epitopes after pagal treatment, which also revealed T-cell epitopes. Of these various epitopes, we note that DTR and STAP are considered to be T-cell epitopes in a Rothbard-type analysis (13). DTR is unlikely as it is "exposed" (on antibody-based tesaats; Table 2); possibly STAP is the T-cell-reactive epitope, although the others (RPAPGS, VTSA, and RPAP) cannot be excluded. However, these speculations are based on the assumption that antibodies and CTL recognize the same site, which is unlikely (43). We are currently examining these epitopes by using peptides and examining whether the CTL produced are H-2 class I restricted.

At this time, our studies indicate that peptides (either as synthetic, as fusion proteins, or as natural mucin) induce humoral rather than cellular immunity, and we are now altering their immunogenicity so that they primarily induce a significant cellular immunity (Table 3). This can be attempted by the use of various immunizing regimes known to bias the immune system towards cellular immunity and also with the knowledge that the T-cell responses generally fall into TH_1 CD4 and TH_2 subsets, the latter being more involved in antibody production and being stimulated by cytokines IL-4, IL-6, and IL-10. Cellular immunity seems to be the product of the TH_1 subset involv-

[5] V. Apostolopoulos, G. A. Pietersz, and I. F. C. McKenzie, unpublished data.

[6] B. R. Acres, M. Hareureni, J-M. Balloul, and M-P. Kieny, personal communication.

ing the cytokines IL-2 and IFN-γ and is down-regulated by IL-10, and to a lesser extent, several other cytokines. With the knowledge of the murine immune response and its relevance to human tumors, satisfactory immunization of mice may be a prelude to the use of synthetic antigens or recombinant products in patients with MUC1$^+$ tumors.

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